IDENTIFICATION OF DESERT HEDGEHOG EXPRESSION IN THE MURINE PALATE AND THE EFFECTS OF EXOGENOUS RETINOIC ACID AND CITRAL ON DESERT HEDGEHOG EXPRESSION.

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INTRODUCTION

The development of the craniofacial complex has sparked intense research efforts, in attempts to define the molecular cascade of events responsible for facial morphogenisis. The driving force of facial morphogenisis, whether from the ectoderm, neural crest or mesenchyme, is still heavily debated (Noden 1988) (Thorogood 1988)(Helms et al. 1994). The importance of the regulatory role these cell populations play during the induction of craniofacial development, may very well be dependant upon the developmental stage or time they are studied (Helms et al. 1997) (Hu et al. 1999) (Kollar et al. 1969) (Kollar et al 1980). Past research has, in fact, proven that the influence of the mesenchyme, neural crest cells or epithelial cells on morphogenesis varies greatly, depending on the precise developmental stage studied (Helms et al. 1997) (Kollar et al. 1980). Transplant studies have demonstrated that both the epithelium and the mesenchyme carry the necessary genetic potential to drive morphogenisis, but are in fact, spatially and temporally dependant (Helms et al. 1994) (Hu et al. 1999). Identification of the genes, receptors, and morphogens responsible for the initiation or induction of facial pattern development points to a complex intricate process involving multifactorial genes and their products (Thesloff et al. 1995). Several gene families and multiple morphogens have been identified as playing critical roles in craniofacial development. The Hedgehog gene family and their receptors, as well as, the Hox genes have been recently implicated as regulatory genes driving craniofacial pattern development (Conlon et al. 1995) (Fienberg et al. 1987) (Young et al. 2000) (Hu et al.1999)

(Hunt et al. 1991). Shh has been identified in the epithelium of the frontalnasal process and maxillary processes (Hu et al. 1999) (Helms et al. 1997), while Dhh expression has been located in the MEE of the mouse palate (Wickson et al. 1996). Ihh transcripts are also expressed during facial development, inducing signals in the developing mandible and mandibular condyle (Kronmiller et al.1996) (Hartman et al. 1997). The well studied, morphogen, retinoic acid has been linked directly and indirectly to the regulation of these genes, but the exact molecular mechanism of control has not yet been clearly defined (Ogura et al. 1996) (Wedden et al. 1991) (Young et al. 2000) (Helms et al. 1997) (Helms et al. 1994). The goal of our research is to evaluate Dhh expression in the murine palate and describe its relationship with the retinoic acid. We hope these results will provide insight to the molecular cascade of events responsible for palate fusion.

LITERATURE REVIEW

In order to properly diagnose and treat craniofacial malformations, detailed knowledge regarding the mechanisms responsible for the developmental defects is essential. The formation of the craniofacial skeletal involves the coordination and integration of the first, second and third brachial arch (Ten Cate 1985). In the mouse the migration of neural crest cells begins at about gestation day eight and continues to day ten (Abbott et al. 1989), and are thought to be responsible for the initial formation and proliferation of the primitive facial pattern (Ten Cate 1985). The skeletal and connective tissue patterns of the face are derived from neural crest cells originating from the margin of the midbrain and hindbrain (Noden et al. 1986,1983) (Young et al. 2000) (Osumi-Yamashita et al. 1990) (Osumi-Yamashita et al. 1994). During early development the craniofacial complex consists of five primordia, which are the recipients of site-specific neural crest cell migration and hence the active areas of proliferation in the primitive facial complex. The five, ectomesenchymal growth regions consist of the frontonasal process, two mandibular processes and two maxillary processes (Ten Cate 1985). The growth and complete fusion of these processes relies on critical timing and intricate reciprocal cell-to-cell interactions between the epithelium and mesenchyme. These facial primordia develop through different morphogenetic mechanisms from separate neural crest cell populations, and therefore regionally react differently to molecules and morphogens during coincident stage development (Young et al. 2000) (Helms et al. 1997).

The formation of the palate is a well-studied process, due to the high percentage of birth defects. Cleft lip and palate has been estimated to comprise 65% of all craniofacial abnormalities, resulting in about one cleft birth per 700 to 1000 births (Ferguson 1988) (Young et al. 2000) (Chenevix-Trench et al. 1992). Children born with cleft palate inherit the physiological, psychological and nutritional deficiencies that accompany the defect. These observations have stimulated immense research efforts to define molecular clefting mechanisms (Young et al. 2000) (Ferguson 1988) (Schneider et al. 1999). The development of the palate has proven to be a highly complex and time specific process, involving reciprocal epithelial - mesenchymal cell interactions.

The palatal shelves of the mouse begin formation as outgrowths of the maxillary processes at about day 12 from mesenchymal condensations, soon after the migration of neural crest cells and their interaction with the ectoderm of the maxillary processes (Shuler et al. 1995) (Ferguson 1981,1988) (Ignelzi et al. 1995). Two peaks of DNA synthesis have been recorded with palatal shelf formation (Ferguson 1988). The first peak occurs just after neural crest cell migration and is responsible for the initial growth of the shelves. The second DNA synthesis peak has a temporal correlation with the vertical development of the shelves. Initially, the palatal shelves grow vertically along the side of the tongue, but rapidly elevate at a precise time to a position above the tongue (Ten Cate 1985) (Shuler et al. 1995) (Ferguson 1988) (Greene et al. 1976). It is important to mention that growth in the width of the head at this time has ceased and only vertical growth continues which, assists shelf elevation (Ferguson

1988). The process of shelf elevation is thought by Ferguson (1988) to be a multifactorial intrinsic force. The force is thought to be intrinsically generated from within the shelf by a significant increase in concentration of glycosaminoglycans. The associated hyaluronic acid possesses an electrostatic charge, which is capable of binding large amounts of water. The synthesis of hyaluronic acid is believed to be to the result of an increase in epidermal growth factor (EGF) expression (Turley et al. 1985). The increase in water decreases mesenchymal cell density and forces the shelves above the tongue. This superior placement allows the epithelial edges of the two opposite shelves to approximate. The one to two, cell layer thick epithelial edge is termed the medial epithelial edge (MEE), and is a vital anatomical element for absolute palatal fusion. The binding of the opposite epithelium layers is very specific, only adhering to each other and not neighboring epithelial cells. The MEE cells form a pseudo-seam through sticky cell surface adhesion with the aid of desmosomes. Complete fusion requires a proper mesenchymal - mesenchymal integration. The sequence of events leading to the disruption of the MEE cells is still in debate, as will be discussed, but a noted cessation of DNA synthesis occurs within the MEE 24 - 36 hours prior to contact (Ferguson 1988) (Shuler 1995) (Greene 1976). Many authors believe that the cessation of DNA synthesis is the initiation of the active cell process, programmed cell death (PCD) by the epithelial cells, in order to, expose the mesenchymal components required for complete fusion (Ferguson 1988) (Shuler 1995). Ferguson has outlined five stages in development, in which failures are more likely to result in cleft palate:

1) Defective palatal growth, which may be caused by improper neural crest cell migration

2) Delayed or failed shelf elevation

3) Defective shelf fusion

4) Failure of MEE PCD

5) Post-fusion rupture

The exact mechanisms of Ferguson's listed possibilities are not known, but the fate of the MEE has lead to several theories, and will be our main focus (Ferguson 1988).

The disruption of the MEE is essential for the proper fusion of the opposite palatal shelves, providing mesenchymal union. The terminal fate of the MEE cells has been argued over the past few decades (Fitchette et al. 1989) (Shuler 1995) (Greene et al. 1974,1976) (Carrette et al. 1992). Even before the MEE cells meet at the midline, very precise temporal and spatial reciprocal cell-to-cell interactions occur that initiate the disruption of the MEE (Shuler 1995). Shuler (1995) describes PCD, migration and transformation as three possible fates of the MEE cells. Many authors believe that the increase in lysosomal enzymes in the MEE, as well as the correlated degradation of epithelial organelles provide significant evidence for programmed cell death (PCD). PCD is activated by the expression of growth factors inducing genetic instructions for the epithelial cells to expire. The MEE cells stop incorporating labeled thymidine 24 hours prior to palatal shelf contact indicating a cessation in DNA synthesis and cell

preservation, providing even more evidence for PCD. Greene and Pratt (1976) found that the addition of cycloheximide, a protein synthesis inhibitor, terminated the active process of programmed cell death. This supported the theory that the MEE disruption was carried out by PCD, since PCD is an active cellular response requiring specific proteins for the initiation and completion of the process (Shuler 1995). Although the inhibition of the proteins prevented PCD, it does not explain how the proteins are transcribed, since DNA synthesis has supposedly ceased in the MEE. However, it is possible that the production of proteins and growth factors responsible for PCD is generated in the mesenchyme (Ferguson 1988) (Shuler 1995). Ferguson describes several growth factors (EGF, TGF, PDGF) that may arise from within the mesenchyme that act directly or indirectly on the MEE cells (Ferguson 1988). The increase in cAMP prior to MEE contact supports the theory of an active cellular process. Fitchett and Hay (1989) describe a second possibility of MEE fate, involving the process of transformation. Cell labeling experiments indicate that the original MEE cells still exist after MEE disruption, but serve a different function. Cells on the superior aspect of the MEE may differentiate into nasal epithelium (Gui et al.1993), while cells located inferior within the MEE may differentiate into oral sqamous epithelium. The MEE cells located in the center of the junction would transform into mesenchymal cells (Fitchett et al. 1989) (Shuler 1995). A third possibility is epithelial cell migration, in which cells of the MEE only migrate to other epithelial cell populations (Carrette et al. 1992). It may be possible that the disruption of the MEE may be a combination of PCD, migration and transformation, depending

upon cell location and the stage of development (Martinez-Alverez et al. 2000) (Shuler 1995). Regardless of cellular fate the production of mesenchymal union of the palatine shelves is inhibited by MEE preservation. What are the molecular mechanisms that initiate the intricate cellular interactions between the epithelium and mesenchyme? The discovery of the genes and their products responsible for the disruption of MEE and subsequent mesenchymal union will provide insight to the cascade of events resulting in an intact palate. Only by studying these mechanisms can we begin to better understand how the defect can be corrected.

Retinoic acid, a vitamin A derivative, has been identified as a powerful morphogen with profound developmental effects on craniofacial morphogenesis (Abbott et al. 1989,1990.1991) (Denker et al.1991) (Wedden 1987) (Helms et al 1996) (Beeman et al. 1994) (Kronmiller et al. 1995). Physiological and pharmcologic doses of retinoic acid initiate and alter mechanisms responsible for facial patterning, proliferation and differentiation (Wedden 1991) (Helms et al. 1997) (Conlon et al. 1995). The vast control retinoic acid has on craniofacial morphogenesis proceeds through the transcriptional regulation of downstream genes, both directly and indirectly (Fisher et al. 1995). The developmental effects of RA are dependent on the spatial and temporal expression of targeted downstream genes, which explains the variety of experimental results involving exogenous RA exposure at discrete developmental stages (Fisher et al.1995). Beeman and Wedden have shown that RA can independently modulate gene expression in the maxilla and mandible at different developmental stages

(Beeman et al. 1994) (Wedden 1991). Wedden claims that RA is not expressed in the chick and mouse mandible, but Kronmiller identified RA expression in the mouse mandible later in facial development and described its effects on odontogenesis (Beeman et al. 1994) (Kronmiller et al. 1995). Retinoic acid induces its developmental control through a combination of nuclear and cytoplasmic receptors, which then regulate gene transcription downstream of RA. These receptors have been classified in the retinoid/thyroid receptor family each linked to multiple functions (Fisher et al. 1995). These ligand-activated transcription factors are controlled by two families of retinoic acid receptors (Conlon et al. 1995). RAR's have been identified as binding 9-Cis-RA and all-Trans-RA with three receptors types alpha, beta and gamma. RXR's, the second RA nuclear receptor, also has three receptors alpha, beta and gamma, but only show high affinity for 9-Cis-RA (Morris-Kay 1993) (Fisher et al. 1995). The importance of retinoic acid became even more relevant with the discovery that these receptors exist in both homo and heterodimer form. The multiple combinations of the dimers then bind to responsive elements on the DNA strands, thereby increasing the multifactorial complexity of retinoic acid morphogenetic potential (Morris-Kay 1993) (Fisher et al. 1995) (Conlon et al. 1995). The identification of ligand-receptor complexes binding to retinoic acid response elements (RAREs) has been linked to homeobox gene expression. RARE's have been located in three homeobox genes (Marshall et al. 1992) (Morris-Kay 1993). Therefore, retinoic acid may have some control over homeobox genetic expression, which has been linked to neural crest cell

migration control (Conlon et al. 1995) (Wedden 1991) (Mackenzie et al. 1991) (Kessel et al. 1991) (Fienberg et al. 1987). Neural crest cells appear to be responsible for early facial patterning and brachial arch development. Slight disruption of this cellular migration pattern results in severe facial malformations and clefts (Dencker et al. 1991) (Ten Cate 1985) (Noden 1988). Therefore, differential expression of RA nuclear receptors and homeobox genes can have dramatic effects on the development of the maxillary processes and hence the palatal shelves.

The effects of exogenous retinoic acid has on palatal shelf formation varies greatly depending on the dose and the stage of shelf development (Abbott et al. 1989,1990). The mechanisms producing cleft palate and the severity of the cleft involved are dependent on the developmental stage (Abbott et al. 1989,1990). During the eighth day of gestation the neural crest cells in the mouse begin to migrate to the region of the future first brachial arch and initiate the patterning of the maxillary primordia and the palatal shelves. An increase in exogenous retinoic acid exposure at gestation day eight dramatically inhibits appropriate neural crest cell migration, which results in reduced palatal shelf size (Abbott et al. 1989). Exogenous retinoic acid exerts an alteration in transcriptional control by binding nuclear receptors and altering Hox gene expression (Mackenzie et al. 1991). After day ten neural crest cell migration to the palatal shelves is complete in the mouse. Exogenous retinoic acid exposure at pharmcologic doses at day ten influences the size of the shelves, by reducing mesenchymal cell proliferation (Abbott et al. 1989,1990). These smaller shelves

are unable to contact after vertical shelf growth, resulting in a cleft. As development continues, exogenous application of RA, at days 13, 14 and 15 influences the phenotype and fate of the medial epithelial edge, which is the main focus of our research.

As previously described, the fusion of the two opposite palatal shelves is critically dependent upon the morphologic changes that occur within the MEE prior to and during shelf contact (Martinez-Alvarez et al. 2000), (Ferguson 1988), (Greene et al. 1991) (Shuler 1995). The eventual disappearance of the MEE appears to be under multifactorial control. Although, the number of epithelial cells in the MEE is small and appears insignificant to the development of the palate, these epithelial cells control the rate-limiting step for complete mesenchymal fusion (Shuler 1995).

The teratogen retinoic acid mediates the fusion of the palate by altering the expression of multiple growth factors, within the MEE, responsible for proliferation and differentiation (Schneider et al. 1999) (Abbott et al. 1988,1990). Palates exposed to exogenous retinoic acid at days 14 and 15 demonstrated a maintenance or survival of the MEE and reduced programmed cell death (Abbott et al. 1990 1991). Abbott's research comparing epidermal growth factor (EGF) and exogenous retinoic acid demonstrated that retinoic acid has similar effects as pharmcologic doses of EGF, leading Abbott to believe RA controls EGF expression. Retinoic acid appears to not only alter the expression of the EGF receptor, but exogenous RA also prolongs the binding of EGF with its own receptor, thereby increasing the duration of EGF action. The increase in EGF

activity results in medial epithelial edge cell proliferation and overall MEE survival. Again, this epithelial seam survival inhibits proper epithelial-mesenchymal interactions and induces abnormal spatial and temporal cell differentiation (Young et al. 2000) (Shuler 1995) (Greene et al. 1991).

Retinoic acid is now known to influence the expression of multiple genes, but the exact molecular cascade and all the genes involved under retinoic acid manipulation are not fully understood. Altered gene expression has been shown to vary greatly depending on exogenous RA dose, as well as temporal and spatial exposure (Abbott et al. 1990) (Young et al. 2000). Experiments that differ slightly in developmental stage, as well as dose produce dramatically diverse results by activating or inhibiting independent genetic cascades. The variety of phenotypic outcomes indicate a much more complex system than previously thought. The epithelium is a key component in the morphognenisis of the face, therefore it seems likely that retinoic acid, with its target of influence the epithelium, would have a major impact on craniofacial development. Exogenous RA treatment on chick embryos results in a deficient frontonasal mass and missing upper beaks (Wedden 1991,1988). The dramatic influence retinoic acid has on the epithelium has prompted researchers to locate the genes responsible for proliferation, differentiation, and pattern formation in the facial complex utilizing retinoic acid as a genetic tool. Pharmcologic doses of retinoic acid has the potential to facilitate polarizing activity in the limb (Helms et al. 1996) (Ogura et al 1996). The identification of exogenous RA polarizing potential in the limb

bud has introduced a correlation between the Hedgehog gene family and RA (Helms et al. 1994).

The Hedgehog gene family consists of three known vertebrate genes: Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog (McMahon 2000) (Tate et al. 2000) (Kamisago et al. 1999). Although, three genes have been identified in vertebrates the majority of research has focused on Shh due to its immense potential to regulate anterior-posterior patterning (Hammerschmidt et al. 1997) (McMahon 2000) (Helms et al. 1997) (Hu et al. 1999) and midline structures (Ingham 1998). Ihh and Dhh gene expression has been identified in cartilage formation and nervous/reproductive systems, respectively. Although, very little attention has been dedicated to Ihh and Dhh, recent research has exposed an extremely complex Shh signaling cascade involving multiple proteins, receptors, second messenger systems, and transcription factors (Ingham 1998) (Britto et al. 2000) (Johnson et al. 1998) (Walterhouse et al. 1999) (Weed et al. 1997).

Sonic Hedgehog is believed to be inactive until it is proteolytically cleaved and modified by the binding of cholesterol (Porter et al. 1996) (Porter et al. 1995). Cholesterol binding to the cleaved Shh products creates the active signaling molecule (Weed et al. 1997). Diseases involving deficient cholesterol, such as in Smith-Lemli-Opitz syndrome result in similar symptoms as abnormal Shh expression (Roessler et al. 1996) (Chiang et al. 1996) (Walterhouse et al.1999). Auto proteolysis divides Shh into two subunits: a 19K-N-terminal and a 25K-C-

terminal. The N-Hh domain appears to be involved in the signaling cascade, while the C-Hh is responsible for processing (Weed et al. 1997).

It is currently believed the active Shh molecules bind to the receptor Patched (Ptc), a twelve-member domain multipass membrane protein (Marigo et al. 1996). In the absence of Shh the Patched receptor is thought to inhibit Smoothened function, a seven pass membrane protein (Alcedo et al. 1996). Smoothened is required to further transduce the Shh signal (Chen et al. 1996) (Murone et. al 1999) (Denef et. al 2000) (Hammerschmidt et al. 1997). The binding of the Shh protein to the Patched receptor (Ptc) induces a conformational change within the receptor, which relieves the direct inhibition of Smoothened (Chen et al. 1996) (Murone et al.1999) (Traiffort et al. 1998). The binding of Shh to Ptc may also activate Smoothened through a phosphorylation mechanism. As Shh binding continues Ptc is internalized and Smo expression is upregulated. This increases the concentration of Smo at the cell surface, which potentiates the Shh signaling cascade (Walterhouse et al. 1999) (Chen et al. 1996). Smo therefore, may always be present in an active form, but is either directly or indirectly inhibited by Ptc until Shh binding (Stone et al. 1996) (McMahon 2000). Studies have shown that mice with abnormal Ptc receptors result in overactive Shh signaling. The abnormal Shh expression produces phenotypes with neoplastic diseases and syndromes (Chiang et al. 1996) (Roessler et al. 1996) (Belloni et al. 1996). This confirms Ptc's inhibitory regulation of Smo. Hedgehog ligand-dependant signaling appears to have both short and long-range capability (McMahon 2000) (Drossopoulou et al. 2000). Covalently coupled cholesterol

binding may assist Hh function and enhance the ability of Hh to produce long-range signals (Drossopoulou et al. 2000) (Weed et al. 1997). After the inhibitory factors on Smo are released by either direct conformational change of Ptc or indirectly by a second messenger or protein kinase cascade activation, the transcription factor Gli is activated to induce gene expression. Three Gli transcription factors, Gli 1, Gli 2 and Gli 3 have been described, all of which are closely associated with the cellular cytoskeleton (Walterhouse et al. 1999) (Hammerschmidt et al. 1997) (Alcedo et al. 1996) (Alcedo et al. 1997) (McMahon 2000). The transduction of the Smoothened signal that modulates the Gli complex is not fully understood, but recent research has implicated protein kinase and G proteins as modulators (McMahon 2000) (Ingham 1998). Gli is associated with several molecules forming the Gli-fused-Cos2 complex, which is bound to microtubules of the cytoplasmic cytoskeleton (Denef et al. 2000) (Johnson et al. 1998). The events leading to the release and activation of Gli from the complex have not been clearly defined. Gli, however, does appear to be the molecule responsible for delivering the Shh message to the nucleus (Johnson et al. 1998) (Weed et al. 1997). The recently discovered gene Dispatched adds even more complexity to the Shh signaling story (Burke et al. 1999). Dispatched expression is found only in cells that release Hh, not in cells receiving the Hh signal (Burke et al. 1999). Blocked expression of Dispatched in Hh producing cells results in the normal production of Hh levels, but fails to release Hh from the cell surface. Hh then accumulates in the posterior compartment of the cells (Burke et al. 1999). Therefore, Dispatched may be

responsible for modifying Hh and preparing Hh for its extracellular release by carrying it to the cell surface (Burke et al. 1999) (McMahon 2000). Hh signaling is further complicated by the modulation of Hedgehog interacting protein (Chuang et al. 1999). Hip is also a transmembrane protein that binds to Hh proteins with similar affinity to that of Ptc. Hip expressing cells have been identified next to Hedgehog producing cells. Hip therefore, may alter or modulate Hh signaling by producing another feedback loop (Chuang et al. 1999). Hip appears to interact with all three Hh genes: Desert, Indian and Sonic (Chuang et al. 1999). Researchers have also noted that Hh signaling activity can be independent of Hh/Ptc binding suggesting the possibility of multiple Hh receptors (Johnson et al. 1998).

Patch 2, a second Hh receptor has been identified, and implies differential genetic expression of Hh (Motoyama et al. 1998). Both Ptc 1 and Ptc 2 are modulated by Shh, as well as, down regulated by Gli 2, indicating some conserved regions (Motoyama et al. 1998). Some researchers believe Ptc 2 is not expressed in cells that receive the Hh signal, but is only expressed in cells that produce the Hh signal (Johnson et al. 1998) (Murone et al. 1999). Biochemical analysis of both Ptc 1 and Ptc 2 has concluded that both bind to all three Hh members with similar affinity, and all three, form complexes with Smo (Carpenter et al. 1998). It is interesting to note that the spatial expression of Ptc 1 and Ptc 2 do not coincide. Ptc 1 is expressed in multiple cell populations throughout the mouse embryo, while Ptc 2 is spatially restricted limiting expression in the skin, PNS and reproductive system (Carpenter et al. 1998). As

development, suggesting abnormal neural crest cell function (Ahlgren et al. 1999) (Chiang et al. 1996). The reduced Shh signal resulted in a decrease in neural crest cell survival, which lead to abnormal head size and brachial arch structure (Ahlgren et al. 1999). Therefore, Shh plays two roles during development. First, in early development Shh modulates pattern development and symmetry. Second, later in development Shh is responsible for cellular proliferation and maintaining cell survival and function. Similar to retinoic acid, Shh is site and stage specific (Hu et al. 1999) (Helms et al. 1997). The control of cellular proliferation and survival, Shh expression and its related gene Dhh has on development can be applied to the characterization of the MEE and its eventual fate. The identification of Dhh transcripts and speculation on the possible role Dhh plays in the MEE during palatal fusion is the focus of our research.

The discovery that Shh and retinoic acid induces polarizing centers in the chick and mouse limb bud has influenced researchers to search for such organizing centers of growth in the craniofacial complex (Hu et al. 1999) (Helms et al. 1997). Shh is expressed exclusively in the epithelium of the frontalnasal and maxillary processes (Helms et al. 1997). Transplantation of these Hh expressing epithelial cells into the mouse limb bud has proven to stimulate polarizing activity and digit duplication (Helms et al. 1994). Interruptions of the Hh signaling pathway by retinoic acid produces severely altered frontalnasal and maxillary processes, resulting in midface abnormalities (Helms et al. 1997). Excess Shh expression causes Hypertelorism. It can, therefore, be concluded that the epithelium plays a stage-specific organizing role in craniofacial patterning

(Helms et al. 1997) (Hu et al. 1999). In other words, the ability of the epithelium to induce pattern formation is location-dependant as well as stage-dependant. Certain epithelial cell populations have varying organizing capabilities (Drossopoulou et al. 2000) (Morgan et al. 1998). The Shh signal appears to be generated from within the epithelium (Helms et al. 1997). Retinoic acid control of the Shh signaling pathway of the frontalnasal and maxillary process are also both dose and time dependant (Riddle et al. 1993) (Helms et al. 1997).

As already mentioned defects or alterations in the Shh signaling mechanism can result in severe craniofacial malformations, such as Holoprosencephaly, Cyclopia, facial clefts and other axial patterning defects (Chuang et al. 1999) (Belloni et al. 1996) (Roessler et al. 1996) (Helms et al. 1997). Defects in Hh receptors such as Ptc or other cascade mechanisms, like Gli, result in Basal Cell Nevus syndrome and other neoplastic diseases. There has been an immense effort by the scientific community to described the Shh signaling pathway and it's contribution to the complex process of facial growth and development, but very little research has focused on the other two Hh family members: Indian and Desert.

Indian Hedgehog is closely associated to the process of skeletal development (Karp et al. 2000) (St. Jacques et al.1999) (Yang et al. 1998). Ihh null mice mutants display reduced chondrocyte proliferation and diminished endochondral bone formation (Karp et al. 2000) (St. Jacques et al.1999) (Yang et al. 1998). Ectopic Ihh expression results in Preaxial Polydactyly, by interfering with the Ptc-Gli pathway. It appears that the related genes Ihh and Shh utilize

similar pathways for transcriptional expression (Yang et al. 1998) (McMahon 2000). Ihh also plays a significant role in facial development with its expression located in the mandible and mandibular condyle (Hartman et al. 1997) (Kronmiller et al. 1996).

Desert Hedgehog expression has been recorded in both the male reproductive system and the peripheral nervous system (McMahon 2000) (Bitgood et al. 1995) (Bitgood et al. 1996). More specifically Dhh expression is localized in the Sertoli cells of the testis, Schwann cells and avascular endothelium (Parmantier et al. 1999) (Mirsky et al. 1999) (Clark et al. 2000). The Dhh signal appears to be responsible for the differentiation of reproductive cells, sperm and the development of the perineurim, which pertains to our research (Matsui 1998) (Parmantier et al. 1999).

The Dhh signaling cascade contributes to the formation of the connective tissue nerve sheath, known as the perineurium, located around peripheral nerves (Parmantier et al. 1999). This sheath serves as protection for the peripheral nerves, as well as, a specialized communication organ or barrier to extracellular nerve surroundings. This barrier mediates proper cell-to-cell interactions allowing proper nerve function and survival (Salzer 1999). Dhh-null mice have abnormal peripheral nerve sheaths. Parmantier et al. (1999) observed reduced collagen, a thin and disorganized perinerium, an inconsistent basal lamina, absent Connexin 43, and abnormal perineural tight junctions, which resulted in the improper diffusion of proteins and immune cells (Mirsky et al. 1999) (Parmantier et al. 1999). Connexin 43 is responsible for mediating inter and intra

cellular interactions. The cell surface proteins and associated cellular interactions of the perineurium may be analogous to the desmosomes and gap junctions of the periderm or MEE of the developing palatal shelves. Dhh expression has been identified in the MEE in the mouse at day 12 and 14 by in-situ hybridization (Wickson et al. 1996). The expression of Dhh during palate fusion has not previously been recorded, nor has any speculation been made on a possible role Dhh may have on the process of shelf fusion. Recently, TGF-ß3 has shed some light on palate fusion. TFG-ß3 has previously been shown to be responsible for cell proliferation, differentiation, migration and epithelial-mesenchymal transformation (Brunet et al. 1995) (Taya et al. 1999) (Proetzel et al. 1995) (Schneider et al. 1999) (Kaartinen et al. 1995) (Young et al. 2000). TGF-ß3 knockouts produced a maintenance or survival of the MEE (Kaartinen et al. 1997). TGF-ß3 is expressed in the MEE prior to shelf fusion, and may be responsible for the breakdown of the MEE's basement membrane allowing eventual MEE disruption. The breakdown of the basement membrane allows epithelial/mesenchymal cell interactions resulting in molecular migration to initiate cell transformation, migration or programmed cell death. Pharmcologic dose application of TGF-ß3 disrupts the basement membrane in TGF-ß3 null mice and allows proper palate fusion (Kaartinen et al. 1997) (Young et al. 2000).

Hedgehog genes not only play a significant role in cell patterning and differentiation, but also cell survival (Britto et al. 2000). When Shh expression is blocked, the level of programmed cell death increases and vise versa (Oppenheim et al. 1999) (Ahlgren et al. 1999). The increase in Shh also elevates

Gli, which may be indirectly involved in maintaining cell populations by preventing PCD. RA as described, upregulates Hh gene expression, which may prolong MEE cell survival by several molecular mechanisms, including an increase in EGF expression. Ectopic expression of Shh produces prolonged proliferation, but the results vary both temporally and spatially (Oppenheim et al. 1999) (Rowitch et al. 1999) (Kalyani et al. 1988). Oppenheim concluded that Shh can either increase or decrease programmed cell death depending upon not only the developmental stage of the cell, but also at site-specific cell populations (Oppenheim et al. 1999). It is possible that specialized cells exist within cell populations that express different surface receptors at stage-specific times.

The goal of our research was to evaluate Dhh expression in the mouse palate at day 13 to 14. It is during this stage of murine development that the opposing palatal shelves initiate contact. The second aspect of our research was to evaluate the influences exogenous RA and citral has on Dhh expression. We propose that Dhh expression can be localized within the MEE at day 13 and14 and that exogenous application of RA will induce Dhh expression. We believe Dhh may play a significant role in the MEE during palate fusion, providing the necessary genetic molecular mechanisms responsible for seam fusion and possible MEE disruption.

Material and Methods:

Isolation of palatal shelves

Swiss mice (CD-1) (Charles River, Wilmington, MA) were used in the experiment. Day 0 gestation is indicated by the presence of a vaginal plug the morning after mating. Pregnant (day 13) females were confirmed by Theiler's (1989) morphological criteria and sacrificed by CO_2 euthanasia. Embryonic palatal shelves were harvested and rinsed in Hanks balanced salt solution (Sigma, St Louis, MO) and transferred to BGJb medium (Life Technologies, Gaithersburg, MD) supplemented with 100U of Penicillin/ Streptomycin (Sigma, St. Louis, MO) and 50 µg/ml Ascorbic Acid (Sigma, St. Louis, MO).

Retinoic acid treatment of palatal shelves

The tissues were cultured for 18 hours at 37°C in a 5% CO_2 and 95% air incubator. 30 palatal shelves were used in each of the three groups: control (DMSO), all-trans retinoic acid (0.01 µg/ml) and citral (1.75 µM) (Sigma, St Louis, MO). The levels of DMSO in the control group were adjusted to match those in the retinoic acid treated groups.

Histological examination

Following retinoic treatment, palatal shelves were fixed in Zenker's acetic acid fixative for 2 hours. The tissues were dehydrated through a series of ethanol,

cleared in xylene and embedded in paraffin. Palatal shelves were oriented in a frontal plane for serial sections (8 μm) using a microtome (Riechert-Jung 2040 (Deutschland, GR). The sections were cleared through xylene, dehydrated through a series of alcohol and stained with Hematoxylin (Sigma, St. Louis, MO) and Biebrich's scarlet (EM Science, Cherry Hill, NJ). The tissues were examined under a light microscope (Leica, Heerbrugg, Switzerland).

RNA preparation

Immediately following 18 hours of organ culture, explants were frozen in liquid nitrogen and homogenized in 100 μl of guanidium thiocyanate (GIT) buffer. The homogenized tissues were layered onto a $CsCl_2$ gradient and centrifuged at 55,000 rpm at 20°C for 2 hours (Kronmiller et al, 1995) in an Optima TL Ultracentrifuge (Beckman, Palo Alto, CA). After centrifugation, the supernatant were removed and the RNA suspended in DEPC water. The samples were digested with 2U of DNase I (Ambion, Austin, TX) at 37°C for 30 minutes. The reaction was stopped with 0.1mM EDTA pH 8.0. The samples were layered on a $CsCl_2$ gradient and centrifuged. The supernatant was removed and the RNA resuspended in DEPC water. The RNA was precipitated with 0.3M Sodium Acetate pH 6.0 in 70% ethanol and placed into -20 °C degree freezer overnight. The samples were centrifuged at 12,000 rpm, at 4°C for 20 minutes and washed in 70% ethanol. The RNA was air dried for 15 minutes at room temperature to remove any traces of ethanol. The RNA was resuspended in DEPC water and the concentrations were determined using a spectrophotometer (Milton Roy 601,

Rochester, NY).

Quantitative RT/PCR

Total RNA (1µg) was added to a reverse transcription (RT) mixture containing 1X RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol (DTT), 2.5 mM MgCl2, 1 mM deoxyribonucleic acid (dNTPs), 10 mM RNase inhibitor, 15 U MultiScribe MMLV reverse transcriptase (PE Biosystems, Foster City, CA) and 1.5 µM Random hexamers. RT profile was as follows: annealed at room temperature for 10 minutes followed by reverse transcription at 42°C for 20 minutes. One-fifth of the resulting cDNA was added to a standard PCR mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.75 mM $MgCl_2$, 1 mM dNTPs, 100 pmole primers and 2.5 U AmpliTaq Gold DNA polymerase (PE Biosystems, Foster City, CA). The PCR temperature profile was as follows: 94°C for 10 min followed by 40 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec, and primer extension at 72°C for 7 minutes. The Dhh primers were as follows Dhh: 5' primer, 5'-ccgcaaccacatccacgtat-3' (534*553 bp), 3' Primer, 5'-gcgagcagcgaacaccagat-3' (812*831), product size 298). QuantumRNA 18S Internal Standards (Ambion, Austin, TX) was used as an internal control to allow direct comparison between the retinoic acid, citral and control samples. 2ul of a 2:8 ratio of 18S Primer/ Competimer mix was added to each PCR reaction.

Analysis of quantitative PCR data

The PCR products were evaluated during the exponential phase of the

amplification process. One fifth (10 µl) of the PCR product was separated on 1.5% agarose gels and stained with ethidium bromide (10 mg/ml). The gels were scanned and analyzed using the Gel Doc 2000 (Bio-Rad, Hercules, CA). A minimum of three assays were replicated for each data group. The ratio of densities for target Dhh to 18S ribosomal RNA PCR bands were calculated for each reaction and standardized. The PCR products were confirmed by restriction enzyme digestions, Pst I for the Dhh fragment and Spe I for the 18S Competimer fragment. A mean value of ratios was calculated for from the individual assays (3 ratio values for the retinoic acid group, and 6 ratio values for the citral group) and plotted on a bar graph. Standard mean errors and unpaired two tail T-test assuming equal variance were calculated for each of the treatment groups.

RESULTS

Frontal sections through gestational day 13 embryonic mouse heads (Figures 1 and 2) were examined to determine the best method for isolation and removal of the palatal shelves. Upon removal, the shelves (Figure 4) were compared with the *in vivo* shelves (Figures 3). No detectable histological changes were observed. In addition, retinoic acid (Figure 5) and control (Figures 6) palatal shelf explants were compared against freshly dissected palatal shelves (Figure 4) and *in vivo* shelves. No histological changes were observed.

Exogenous addition of retinoic acid increased the relative levels of desert hedgehog (Dhh) mRNA transcripts in all three assays (Table 1). The PCR fragments matched the predicted size for Dhh (298 bp) and 18S Ribosomal RNA (489 bp) (Figure 7). Restriction enzyme digest of Dhh using Pst I produced DNA bands matching the predicted sizes of 210 bp and 89 bp (Figure 8). Digestion of 18S ribosomal RNA band resulted in predicted fragment sizes of 378 bp and 110 bp. (Figure 9). The mean ratios of Dhh to 18S rRNA were calculated (Table 1). Comparison of the retinoic acid treated explants with the control (DMSO) demonstrated a relative increase in Dhh mRNA transcripts by approximately 8.2% (Graph 1). A two tail T-test analysis showed a 95% confidence that the differences between the means for the RA and control groups were statistically significant (Table 2).

Citral (3, 7 dimethyl-2,6-octadienal) is a known inhibitor of retinoic acid synthesis (Schuh et al, 1993) and has been shown to decrease endogenous levels of

retinoic acid in the developing mouse mandible (Kronmiller et al, 1995). In all six assays, citral decreased the relative levels of Dhh transcripts of palatal shelf explants (Table 3). The PCR fragments matched the predicted size for Dhh and 18S Ribosomal RNA (Figure 10). Restriction digests confirmed Dhh and 18S rRNA specific expression in both the citral and control treated groups (Figure 11). The mean ratio densities of Dhh to 18S rRNA were calculated for both groups (Table 3). Analysis of the mean ratios shows a relative decrease in Dhh mRNA transcripts by approximately 10.4 % (Graph 2) in citral treated explants. A two tail T-test showed a 95% confidence these differences were statistically significant (Table 4).

To test if the increase was gene specific, explants were assayed for B *Actin (a housekeeping structural protein) quantitative PCR. No changes in B *Actin transcript levels were detected between retinoic acid, citral and their respective control groups (data not showed).

DISCUSSION

To our knowledge this is the first report of the effects exogenous RA and citral have on the expression of Dhh in the medial epithelial edge of the mouse palate at day 13 and day 14. The expression of Dhh has been exclusively located in the MEE by in situ hybridization at day 12 and day 14 of the developing mouse palatal shelves (Wikson et al. 1996). The fact that the Dhh mRNA transcripts were confined to the MEE, and not seen in the adjacent mesenchyme, suggests Dhh may play a crucial role in the intricate process of initial seam formation and complete fusion of the opposite palatal shelves (Wickson et al. 1996). Dhh, like Shh is exclusively expressed in the epithelium of certain areas of the craniofacial complex (Helms et al. 1997) (Hu et al. 1999) (Young et al. 2000). Palatogenesis has proven to be an extremely complex developmental process involving specific reciprocal epithelial-mesenchymal interactions, which drive the expression of numerous genes and their respective proteins and receptors (Young et al. 2000). These time and site –specific coordinated interactions occur rapidly since palate fusion occurs over a 12-14 hour developmental period (Shuler 1995). Any interruptions in the comprehensive cascade pertaining to palatal fusion, produces drastic phenotypes. Chemicals, pharmaceutical agents, and other morphogens have been well documented to induce clefts, but the aberrant mechanisms remain unknown (Shuler 1995) (Greene et al. 1991) (FitzPatrick et al. 1993) (Abbott et al. 1994). We have solely focused our research to the time of palatal fusion and

more specifically to the medial edge epithelium, by introducing another player, Dhh at day 13 and day 14 to the cascade of events. We also characterized the effects the powerful morphogen, RA and citral (an inhibitor of endogenous RA), has on the Dhh expression. We believe MEE adhesion and its eventual disruption is the rate-limiting step for proper mesenchymal-mesenchymal fusion of the opposite palatal shelves. The fate of the unique epithelial cells of the MEE has created an intense controversy over the last four decades (Shuler 1995) (Ferguson 1998) (Martinez-Alvarez et al. 2000) (Carette et al. 1992), but no one argues over the grave importance these cells and their interactions with the mesnechyme have on complete palatal fusion. Whether the fate of the epithelial cells of the MEE disrupt by programmed cell death, transformation to mesenchyme, or migration; the identity and characterization of the genes controlling these intricate mechanisms are essential to fully understand the developmental process of palatogenesis.

Recent research using TUNEL techniques has shed more light on the fate of the MEE (Martinez-Alverez et al. 2000). Advanced TUNEL techniques by Martinez-Alvarez et al. (2000) has supplied convincing evidence that the fate of these MEE cells is, in fact, multidimensional. Martinez-Alvarez et al. (2000) demonstrated that not all the cells of the MEE die, but rather some survive. Some of the cells do, in fact, undergo apoptosis. Macrophages are signaled and recruited to the area and can be seen throughout the MEE phagocytosing dead MEE cells. The lineage of other MEE cells can also be traced transforming into mesenchymal cells. Migration was not ruled out, but could not be identified using

the TUNEL technique (Martinez-Alvarez et al. 2000). Several growth factors including, TGF-B3, have been suggested to drive PCD and transformation (Kaartinen et al. 1995) (Kaartinen et al. 1997) (Young et al. 2000) (Martinez-Alverez et al. 2000), by disintegrating the basement membrane, but the upstream genes controlling their expression have not been identified. We have identified Dhh mRNA transcripts in the MEE at days 13 and 14 in the murine MEE. We have also shown Dhh expression is under endogenous and exogenous RA control in the MEE at day 13 to 14. This suggests Dhh plays a role in controlling the fate of the MEE cells by influencing downstream targets and possibly instructs the phenotype of the MEE for initial adhesion. The location and possible role of Dhh expression as well as the receptor Ptc 2 is more confined than its relative Shh, which is expressed in multiple locations. Dhh regulation of cell proliferation, survival, and differentiation is constricted to the PNS, reproductive system, and the MEE. This implies Dhh controls more precise cellular processes during development.

The specialized superficial surface and motility of the epithelium during initial contact of the opposite palatal shelves is critical for initial seam formation (Ferguson 1988) (Carette et al. 1992) (Martinez-Alverez et al. 2000) (Fitchett et al. 1989). The motility in the epithelial cells and coincident changes in the cytoskeleton (microtubles) allow the cells to bulge. The bulging of the epithelial cells increases the surface area for sufficient palatal contact, as well as increases the exposure of specialized proteins, desmosomes and other adhesion molecules responsible for the initial seam formation (Martinez-Alverez et al.

2000) (Ferguson 1988). SEM studies have shown the presence of filopodia on the surface of the MEE prior to the adhesion of normal palates (Taya et al. 1999). The genes responsible for the production of these crucial surface proteins and cytoskeleton conformational changes have not been identified, although the growth factors, EGF and TGF, have been implicated (Young et al. 2000). The GLI complex is also associated with the cytoskeleton (Murone et al. 1999) (McMahon 2000).

Dhh has recently been described as one of the genes responsible for the proper development of the perineurium (Parmantier et al. 1999) (Mirsky et al. 1999) (Salzer 1999), or nerve sheath. Dhh expression has been identified in the Schwann cells, which are responsible for the production of the perineurium barrier of the peripheral nerve cells (Parmantier et al. 1999). The perineurium is very similar to the MEE, both relying heavily on specialized cell surface proteins for proper formation, function, and cellular interaction (Mirsky et al. 1999) (Parmantier et al. 1999).

Dhh-null mice in the developing PNS demonstrate phenotypes with abnormal basal lamina, absent gap junction proteins (Connexin 43) and an overall faulty barrier allowing improper molecular and cellular diffusion (Mirsky et al. 1999) (Parmantier et al. 1999). There is an overall reduction in collagen and abnormal specialized compartments within the nerve sheath of the Dhh-null mice (Parmantier et al. 1999). Mirsky and Parmantier both believe Dhh function influences nerve sheath formation, later in development, and is responsible for more than one role during development. Mirsky and Parmantier conclude that

Dhh controls the specialized surface and pattern development of the peripheral nerve sheath, but is also responsible for the transition of mesenchymal cells into the epithelial cells of the sheath. It could, therefore, be hypothesized that Dhh may be responsible for similar developmental processes within the MEE. Like Shh, Dhh may have dual roles, depending upon the developmental stage. Early in MEE development Dhh expression under RA control may induce a molecular cascade responsible for the increased motility of the bulging epithelial cells of the MEE by stimulating cytoskeleton conformational changes. Prior to contact, Dhh expression could encode for the specialized surface proteins required for initial adhesion, since Dhh is responsible for gap junctions, tight junctions and other specialized proteins on the surface of developing PNS cells. Dhh, therefore, may encode for desmosomes and sticky adhesion molecules on the surface of the MEE. After palate shelf adhesion the role of Dhh may change, by either inducing or allowing the transformation of MEE cells into mesenchymal cells. Dhh could provide the stimulation or inhibition of transformation. Evidence has been documented that Dhh has the capability to induce transformation of mesenchymal cells to epithelial cells (Mirsky et al. 1999), so it is feasible that Dhh expression can be associated with the opposite epithelial-mesenchymal transformation. An increase in exogenous RA exposure at day 13 to 14 upregulates Dhh expression, which at this site-specific stage is responsible for proliferation and patterning. An abnormal elevation in Dhh mRNA transcripts could increase and prolong the production of desmosomes and bulging epithelial cells responsible for adhesion, resulting in an abnormal surface phenotype. A

prolonged adhesion stage may delay or alter the time specific progression from epithelial shelf adhesion to mesenchymal fusion, resulting in a cleft. The process of fusion is very rapid and a slight disruption results in drastic consequences (Ferguson 1988) (Shuler 1995).

Dhh expression, like its family member Shh, is stage dependent, capable of more than one developmental role (Helms et al. 1997). After the initial adhesion of the opposite palatal shelves, Dhh expression may switch from the driving force of MEE proliferation and cell surface characterization to cellular differentiation and survival. The effect of exogenous retinoic acid on Shh expression is both time and dose dependent (Helms et al. 1994) (Hu et al. 1999). Precise differences in dose and developmental stage of RA application will produce drastically different results (Helms et al. 1994). Early in MEE contact RA exposure upregulates Dhh expression and may induce MEE cell survival, possibly through an increase in EGF expression (Abbott et al. 1988). After MEE adhesion and the initiation of MEE disruption the application of exogenous RA may alter a completely separate molecular cascade responsible for PCD and cellular transformation. Mirsky and Parmantier concluded Dhh controls mesenchymal- epithelial transformation in the final stages of nerve sheath development. Altered expression of Dhh may inhibit the expression of downstream genes like TGF-ß3, which have been hypothesized to control epithelial-mesenchymal transformation (Young et al. 2000) (Parmantier et al. 1999). Dhh may inhibit or stimulate TGF-ß3 expression depending on the stage. It may be possible that that Dhh initiates the process of transformation and TGF-

ß3 finishes the process. TGF-ß3 expression is also altered by exogenous RA (Young et al. 2000) (Martinez-Alverez et al. 2000), which suggests a link between Dhh and TGF-ß3. Dhh may be upstream of TGF-ß3 and could control its expression either directly or indirectly. Any disruption, by increased RA levels or other molecules, in the Dhh cascade could prevent the time specific process of MEE disruption.

Shh expression has been inversely correlated to the active process of programmed cell death in craniofacial neural crest cells (Ahlgren et al. 1999). A reduction in Shh expression results in increased cell death and vice-versa (Oppenheim et al. 1999). It seems reasonable that the increased Dhh expression could also promote cell survival in the MEE by inhibiting PCD. Exogenous application of RA increases Dhh expression, which then could inhibit PCD and promote MEE cell survival.

The presence and integrity of basement membrane may prevent epithelial-mesenchymal interactions that normally induce migration and transformation of the MEE cells. The basement membrane acts as a barrier preventing mesenchymal signals that induce differentiation. Dhh-null mice demonstrate porous and non-continuous membranes in developing peripheral nerves (Mirsky et al. 1999) (Parmantier et al. 1999), suggesting Dhh produces and maintains the basal lamina of the peripheral nerve sheath (Mirsky et al. 1999). Dhh may also maintain the integrity at the basement membrane of the MEE. Increased Dhh expression by exogenous RA may prolong basement membrane survival and prevent migration and transformation mechanisms,

inhibiting MEE disruption. TGF-ß3 controls the disruption of the basement membrane of the MEE. Exogenous TGF-ß3 can produce basement membrane and MEE disruption in TGF-ß3 null mouse palates (Young et al. 2000) (Proetzel et al. 1995) (Taya et a. 1999). The molecular control between Dhh and TGF-ß3 can only be speculative. Further research exploring the role Dhh plays in the MEE during palatal fusion is clearly indicated. Dhh and ptc 2 knockouts will provide further information regarding the function of Dhh in the MEE. Correlations between Dhh expression and the effects on TGF-ß3 and EGF should also be evaluated in attempts to define possible cascade schemes.

Figure 1

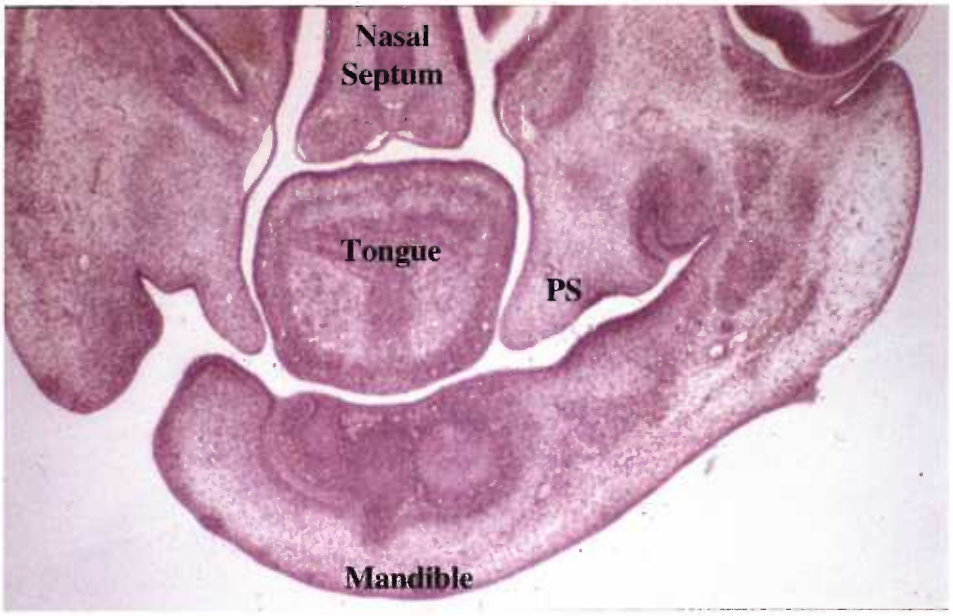


Figure 1 shows a 40x magnification of a frontal section through gestational day 13 embryonic mouse. Major histological structures are labeled, nasal septum, tongue, palatal shelves (PS) and mandible.

Figure 2

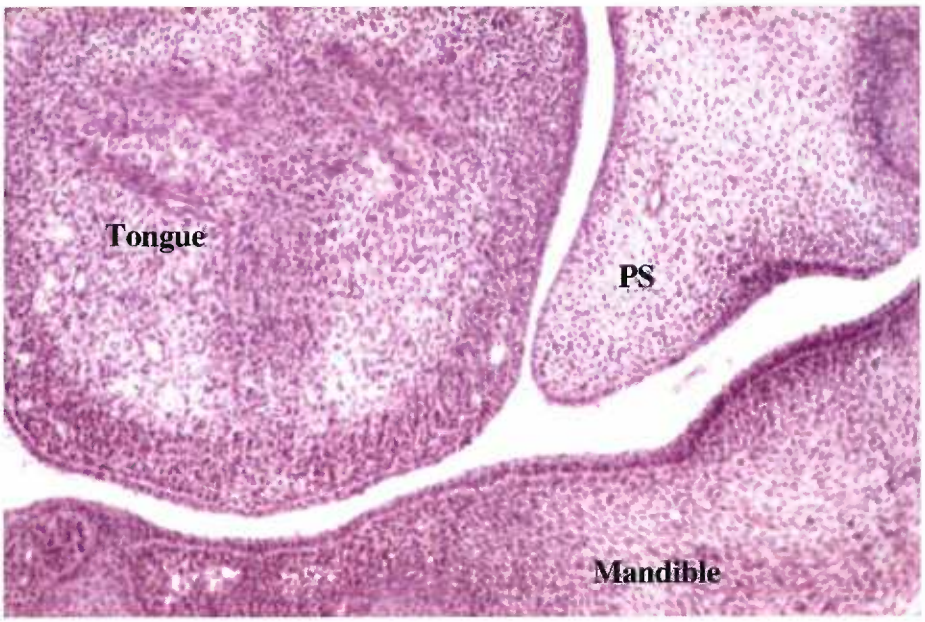


Figure 2 shows a 100x magnification of a frontal section through gestational day 13 embryonic mouse. Major histological structures are labeled, tongue, palatal shelves (PS) and mandible.

Figure 3

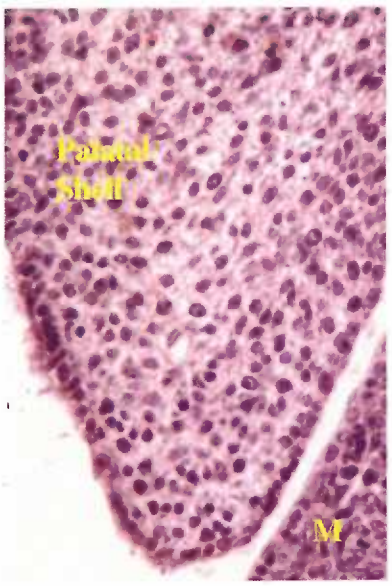


Figure 3 shows a 400x magnification of a frontal section through gestational day 13 embryonic mouse. Major histological structures are labeled, palatal shelf and mandible (M).

Figure 4

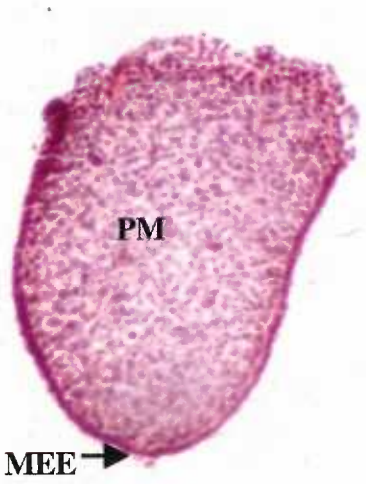


Figure 4 shows dissected gestational day 13 embryonic mouse palatal shelf. The medial edge epithelium (MEE) and palatal mesenchyme (PM) are labeled.

Figure 5

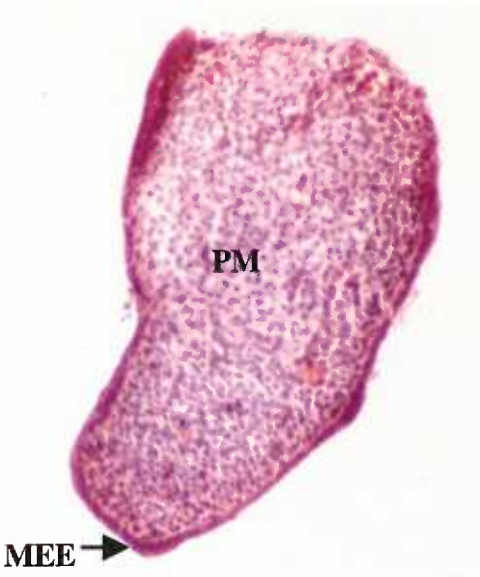


Figure 5 shows a retinoic acid treated gestational day 13 embryonic mouse palatal shelf. The medial edge epithelium (MEE) and palatal mesenchyme (PM) are labeled.

Figure 6

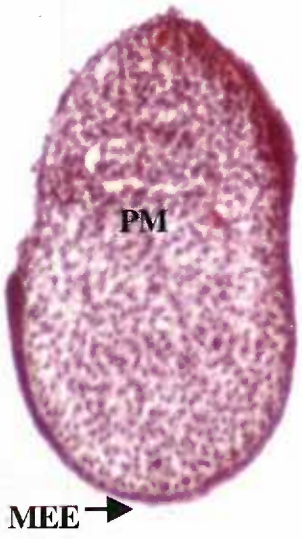


Figure 6 shows a control group gestational day 13 embryonic mouse palatal shelf. The medial edge epithelium (MEE) and palatal mesenchyme (PM) are labeled.

Figure 7

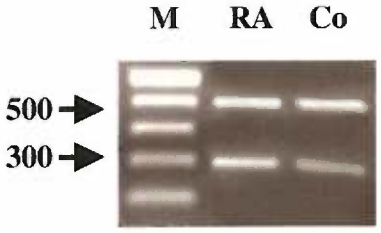


Fig 7. Dhh wild type and 18S rRNA PCR amplification of gestational day 13 embryonic mouse palatal shelves after exposure to retinoic acid in vitro. 1.5% agarose gel electrophoresis of RT/PCR product, 10 µl/lane. RA represents the all trans retinoic acid group while Co represents the control group. The predicted size of Dhh is 298 bp and 18S Ribosomal RNA is 489 bp. Marker lane (M) contains a 100 bp DNA ladder.

Figure 8

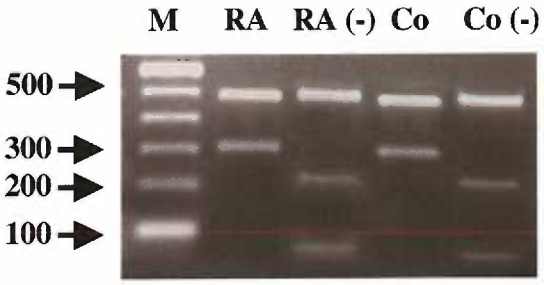


Fig 8. Restriction enzyme digests of gestational day 13 embryonic mouse palatal shelves RT/PCR products after exposure to retinoic acid in vitro. 1.5% agarose gel electrophoresis. RA (-) and Co (-) represent Pst I restriction enzyme digest of retinoic acid and control groups respectively. RA and Co represent undigested fragments. Restriction enzyme digest of Dhh using Pst 1 produced DNA bands matching the predicted sizes of 210 bp and 89 bp. Marker lane (M) contains a 100 bp DNA ladder.

Figure 9

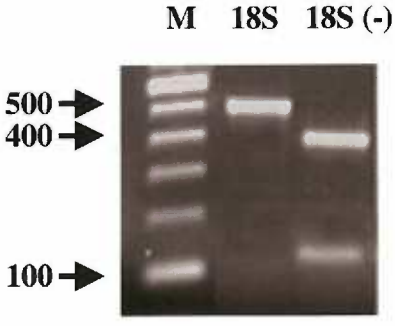


Fig 9. Restriction enzyme digests of gestational day 13 embryonic mouse palatal shelves 18s ribosomal RNA RT/PCR products. 1.5% agarose gel electrophoresis. The undigestion lane is marked (18S) while the Spe I restriction digest is labeled 18S (-). Digestion with Spe 1 resulted in the predicted fragment sizes of 378 bp and 110 bp. Marker lane (M) contains a 100 bp DNA ladder.

Table 1

	RA	Control
Sample 1	0.7992	0.7512
Sample 2	0.759	0.702
Sample 3	0.771	0.7
Average	0.765	0.701
SD	0.00848528	0.00141421
SEM	0.006	0.001

Table 1 shows the ratios of Dhh to 18S rRNA RT/PCR products between retinoic acid and control groups. Three samples are shown with their respective averages, standard deviations and standard mean error (SEM).

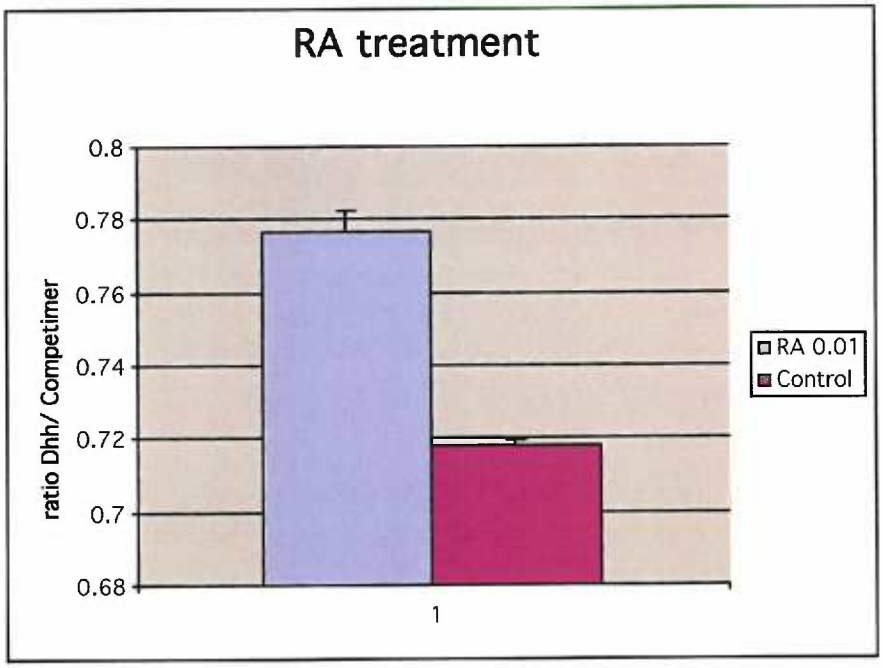
Table 2

t-Test: Two-Sample Assuming Equal Variances

	RA 0.01	*Control*
Mean	0.7764	0.71773333
Variance	0.00042588	0.00084101
Observations	3	3
Pooled Variance	0.00063345	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.85484159	
P(T<=t) one-tail	0.02308467	
t Critical one-tail	2.13184649	
P(T<=t) two-tail	0.04616935	
t Critical two-tail	2.77645086	

Table 2 shows a statistical t-test analysis between the retinoic acid and control assays.

Graph 1



Graph 1 compares the relative ratios of Dhh/competimer between the retinoic acid and control groups. RA shows 8.2% relative increase in transcripts level.

Figure 10

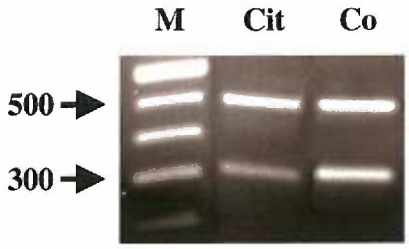


Fig 10. Dhh wild type and 18S rRNA PCR amplification of gestational day 13 embryonic mouse palatal shelves after exposure to citral in vitro. 1.5% agarose gel electrophoresis of RT/PCR product, 10 µl/lane. Cit represents the citral treated group. Co represents the control group. The 298 bp fragment represents Dhh and 489bp fragment is the 18S Ribosomal RNA. Marker lane (M) contains a 100 bp DNA ladder.

Figure 11

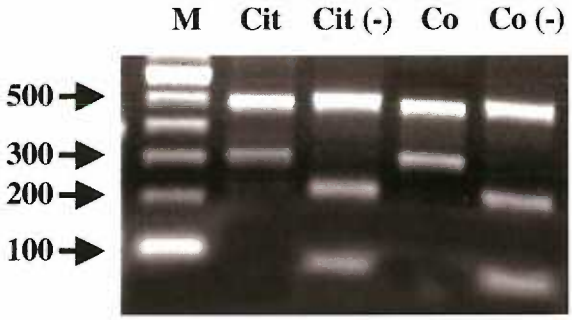


Fig 11. Restriction enzyme digests of gestational day 13 embryonic mouse palatal shelves RT/PCR products after exposure to citral in vitro. 1.5% agarose gel electrophoresis. Cit and Co represent undigested RT/PCR products for the citral and control groups respectively. Cit (-) and Co (-) represent Pst I restriction enzyme digest of citral and control groups. The 298 bp fragment is Dhh and the predicted digest fragments of Pst 1 is 210 bp and 89 bp. The 489 bp fragment is 18S Ribosomal RNA. Marker lane (M) contains a 100 bp DNA ladder.

Table 3

	Citral	Control	Citral/RA
Sample 1	0.6718	0.7209	
Sample 2	0.606	0.7014	0.9251
Sample 3	0.657	0.7222	0.9702
Sample 4	0.7621	0.8377	
Sample 5	0.682	0.7661	
Sample 6	0.6703	0.7226	
average	0.67486667	0.74515	0.94765
SD	0.05050274	0.0500834	0.03189052
SEM	0.02258551	0.02239798	0.018412

Table 3 shows the ratios of Dhh to 18S rRNA RT/PCR products between citral and control groups. Six samples are shown with their respective averages, standard deviations and standard mean error (SEM).

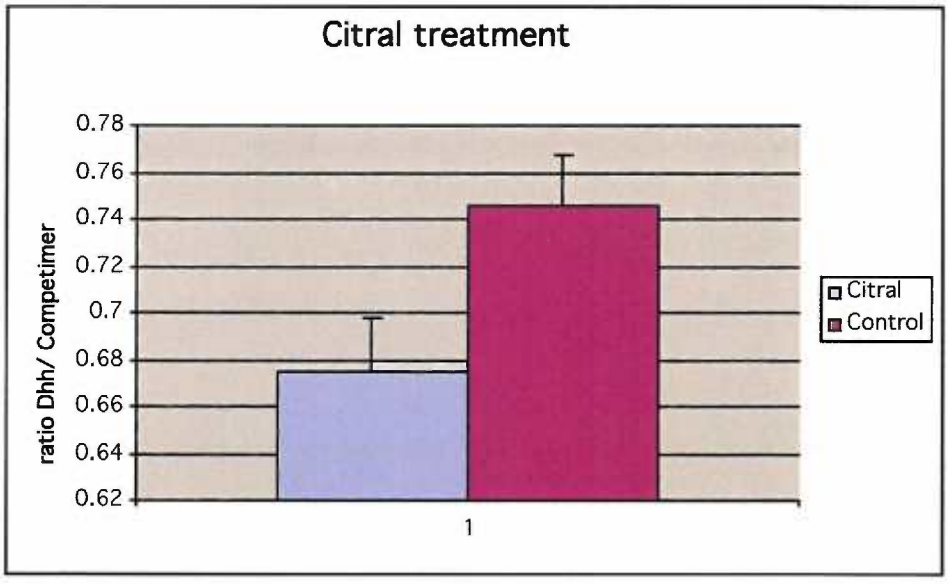
Table 4

t-Test: Two-Sample Assuming Equal Variances

	Citral	*Control*
Mean	0.67486667	0.74515
Variance	0.00255053	0.00250835
Observations	6	6
Pooled Variance	0.00252944	
Hypothesized Mean Difference	0	
df	10	
t Stat	-2.4204775	
P(T<=t) one-tail	0.01801609	
t Critical one-tail	1.81246151	
P(T<=t) two-tail	0.03603217	
t Critical two-tail	2.22813924	

Table 4 shows a statistical t-test analysis between the citral and control assays.

Graph 2



Graph 2 compares the relative ratios of Dhh/competimer between the citral and control groups. Citral exposed groups shows a 10.4% relative decrease in transcripts level.

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